PATENT SPECIFICATION (11) 1 499 846

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(21) Application No. 16509/75 (22) Filed 22 April 1975
(31) Convention Application No. 465 990
(32) Filed 1 May 1974 in
(33) United States of America (US)
(44) Complete Specification published 1 Feb. 1978
(51) INT CL² G01N 33/16 // 21/24
(52) Index at acceptance G1B 11X 17AX 17L 17X2

(54) IMPROVED METHOD FOR ANALYSIS OF ENDOTOXIN-PRECIPITATED LIMULUS LYSATE

(71) We, BAXTER TRAVENOL LABORATORIES INC., formerly Baxter Laboratories Inc., a Corporation organised and existing under the laws of the State of Delaware, United States of America, of One Baxter Parkway, Deerfield, Illinois 60015, formerly of Morton Grove, Illinois 60053, United States of America, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

As has frequently been discussed in the published literature (for example, *Thrombos. Diath. Hemorrage,* Vol. 23, Pages 170—181 (1970), the amoebocyte blood cells of members of the genus *Limulus,* and particularly *Limulus polyphemus,* the horseshoe crab, form clots when placed in contact with pyrogens such as bacterial endotoxin. These amoebocyte cells provide an effective blood clotting mechanism to an injured horseshoe crab, thereby preventing further proliferation and migration of bacteria into other parts of the body.

At the present time *in vivo* pyrogen testing of parenteral solutions is performed in rabbits. Such a test program is very expensive and difficult to operate.

A considerable amount of research has been invested in the use of *Limulus* amoebocytes, after lysing them in water to rupture the cells, as a substitute testing means for pyrogens in sterile products. One typical summary of such recent work with *Limulus* is found in the *Bulletin of the Parenteral Drug Association,* Vol. 27, No. 3, Pages 139—148 (May-June, 1973).

Typically, the *Limulus* amoebocyte cells are lysed by placing them in distilled water, or by any other convenient means for rupturing the blood cells. Following this, the resulting solution is filtered or centrifuged, to remove solids such as cell wall fragments and the like, to yield a protein solution, commonly referred to as *Limulus* lysate.

This protein solution (*Limulus* Lysate) is conventionally used to detect bacterial endotoxin by bringing it into contact with the material to be tested, and observing whether or not a clot of protein is formed which has certain minimum standards of stability. One typical testing standard for stability of the clot is to invert the test tube in which the clot is formed by 180°. If the clot remains intact, a positive endotoxin reaction is recorded. If the clot breaks up, or if no intact clot is ever formed, a negative endotoxin reaction is recorded.

A disadvantage of the clot technique for determining the presence of pyrogens is that the results can vary, depending on how the observer inverts the test tube, and also depending on his subjective interpretation of what constitutes a "clot".

Thus, while the above-described clot-formation technique of analysis for endotoxin is satisfactory for some uses, there are many important and critical endotoxin determinations which must be made, for which a simple clotting type of endotoxin determination has inadequate accuracy and sensitivity to give a sufficiently precise analysis of the presence of endotoxin.

One very important use in this category involves the pyrogen testing of parenteral solutions, as a substitute for the present, expensive and cumbersome live rabbit testing program, which is commonly used by commercial manufacturers of parenteral solutions.

Accordingly, there is a need for a more accurate, quantitative, and sensitive determination in-vitro for the presence of pyrogens such as bacterial endotoxin.

The invention of this application provides a quantitative, improved technique for the determination of pyrogens such as bacterial endotoxin through the use of *Limulus* lysate materials. By use of the method of this invention, a major increase in the sensitivity of detection of endotoxin is achieved, and the concentration of endotoxin present can be quantitatively determined.

In accordance with this invention, there is

provided a method of analyzing a material having an unknown pyrogen content, comprising:—

A) Calibrating a purified solution of portions of *Limulus* lysate, which portions precipitate *Limulus* protein in the presence of a pyrogen, to determine the quantitative sensitivity of the solution to the presence of said pyrogen, and calibration comprising:—

(i) preparing a series of serially diluted dispersions of said pyrogen, wherein a plurality of these dispersions have concentrations of pyrogen of no more than 0.39 nanogram per ml.;

(ii) adding to said series of dispersions a given quantity of said purified *Limulus* lysate solution to form reaction mixtures; and

(iii) thereafter measuring, in a quantitative, photometric manner, the amount of *Limulus* protein precipitated, whereby an abrupt increase in the amount of precipitated protein in one member of said serially diluted series of dispersions when compared with the next more diluted member of said serially diluted series, indicates a positive pyrogen sensing reaction; and

B) Adding to said material said given quantity of a previously unused portion of said purified *Limulus* lysate solution and allowing the reaction mixture to stand under reaction conditions substantially equivalent to the reaction conditions under which purified *Limulus* lysate solution was added to each member of the serially diluted dispersions and thereafter measuring, in quantitative, photometric manner, the amount of protein precipitated in said material from said pyrogen precipitatable portions, whereby the precipitation of a concentration of protein which is at least equal to the concentration of protein precipitated in a said member of the serially diluted series of dispersions in which said abrupt increase in precipitated protein was noted constitutes an indication of a positive reaction to pyrogen in said material of unknown pyrogen content.

Thereafter, one analyzes materials having an unknown pyrogen content by adding to each material the same measured quantity of a previously unused portion of said purified *Limulus* protein solution, under the reaction conditions of Step (ii) above, and thereafter measuring, in quantitative, photometric manner, the amount of protein precipitated in said material from said pyrogen precipitatable portions.

The precipitation of a concentration of protein per ml. of test solution which is at least equal to the concentration of protein precipitated in a member of the serially diluted series of dispersions in which the abrupt increase of Step (c) above was noted is an indication of a positive pyrogen sensing reaction in the material of unknown pyrogen content.

A purified solution of precipitable protein of *Limulus* lysate is typically used, in which excess amounts of extraneous protein which do not precipitate in the presence of pyrogens has been removed. These latter proteins tend to reduce the sensitivity of the analytical results obtained in accordance with this invention. Thus, it is desired to remove by filtration or centrifugation, or other means, the cell wall fragments and other solid portions of *Limulus* lysate, as well as at least some water-soluble, non-precipitable protein fractions. A preferred technique for purification of the *Limulus* lysate protein is shown in the example below, but other purification techniques may be used as desired. Simpler and less effective purification techniques may be used in those cases where results of the high accuracy of Example 1 below are not required.

The serially diluted dispersions of pyrogen used in Step (a) of the calibration step described above may be prepared from commercially available standard endotoxin solution. Typically, a series of successively diluted solutions are prepared having 20 different bacterial endotoxin concentrations, in which the first solution of highest concentration contains 100 nanograms of endotoxin per ml., in which each successive test solution contains one-half of the concentration of the previous test solution, so that the twentieth and last solution contains 0.00019 nanogram of endotoxin per ml.

Following this, a given quantity, preferably the same quantity per vial, of purified, precipitable protein of *Limulus* lysate solution is added to each of the serially diluted pyrogen dispersions, and the dispersions are generally allowed to stand for a period of time, such as 60 minutes, typically at a warm temperature such as 37°C. However, the tests can be effectively practiced with other incubation times and at a relatively wide range of different temperatures, although it is preferred for all of the samples or vials in a test to be processed in a uniform manner.

During incubation, a clot of precipitated protein may appear in vials containing endotoxin of higher concentration, which has been used as a prior indication of positive reaction. However, the present method is considerably more sensitive than a clot indication, and can detect the presence of endotoxin at a much lower concentration.

Following the incubation step, the amount of protein precipitated is measured in a quantitative, photometric manner. This is generally accomplished by centrifuging or filtering the precipitated protein, to separate it from the supernatant. The purpose of this is to remove as much unprecipitated protein as possible from

the solution, to facilitate the quantitative measurement of the amount of precipitated protein remaining.

Following this, the protein can be quantitatively, photometrically measured using, for example, well-known techniques of absorbence, fluorescence, or light scattering.

It is generally preferable to measure the photo-absorbence of a solution of the precipitated protein with Folin reagent in an electromagnetic wavelength range of 450 to 1000 millimicrons, and particularly using the analytical technique described by Oliver H. Lowry, et al. in the article in *Journal of Biological Chemistry*, Vol. 93, pp. 265 to 275 (1951).

Preferably, each aliquot of solution tested contains sufficient precipitatable protein to provide an absorbence of less than 0.2 in the essential absence of endotoxin, and an absorbence in excess of 1 upon complete protein precipitation, at the wavelength observed.

When the precipitated protein samples from the serially diluted dispersions of pyrogen are analyzed photometrically, using an absorbence principle, one can obtain a specific absorbence reading for each sample which correlates with a specific concentration of endotoxin of each known sample.

Thereafter, utilizing the same calibrated *Limulus* lysate material and the same reaction conditions, one can analyze materials of unknown pyrogen content by adding to the material a measured quantity of a previously unused portion of the same purified *Limulus* protein solution, and thereafter measuring in the same photometric manner the amount of protein precipitated. Accordingly, an optical density reading, which is similar to an optical density reading of a specific tube of known endotoxin concentration also measured, is an indication that a similar concentration of pyrogen is present in the unknown material.

Alternatively, the decrease in photoabsorbence in the supernatant solution can be measured, after removal of precipitated proteins, from which the amount of protein precipitated can be determined.

Example.

Atlantic Ocean horseshoe crabs (*Limulus polyphemus*) were collected and placed in a rack to restrain them in a position with their ventral sides facing upwardly. The joint between the first two segments of the crabs (the prosthoma and the opisthoma) was prepared by swabbing with alcohol. The joint was then penetrated with a blood collection needle mounted on the end of a conventional blood bag manufactured by the Fenwal Division of Travenol Laboratories, Inc., Morton Grove, Illinois, but modified so that the blood collection tube was only 5 inches in length. The bag contained 300 ml. of 3 percent (weight/volume in grams per litre) sodium chloride solution, containing 2.87 grams of dissolved ethylenediaminetetracetate, (EDTA).

The horseshoe crabs were bled one by one as necessary until 300 ml. of blood had passed into the blood bag, which had a 600 ml. capacity. The five-inch blood donor tubing was sealed near its entrance to the bag with a dielectric heat sealer (HEMATRON heat sealing unit sold by the Fenwal Division of Travenol Laboratories, Inc.). The blood collection tube was then cut off near the heat-sealed section, to remove the needle.

Two bags, prepared as shown, were selected and balanced as necessary with weights, and then spun in a Sorvall RC 3 centrifuge for seven minutes at a 1,000 Gravity force (about 1,800 rpm.), to cause the amoebocyte cells from the *Limulus* blood to settle. In cases where the blood appears to be sedimenting well, it is sometimes sufficient to only apply a 600 Gravity force (about 1,500 rpm.) for seven minutes.

Following the cell centrifuging step, each blood bag was placed on a 10° inclined plane with the sealed stub of the blood collection tubing pointing downwardly, and the collection tubing was once again opened by cutting. The supernatant was decanted carefully, to leave the settled cells remaining in the bag. Following the decanting step, the collection tubing was once again heat sealed in the manner previously described.

Following this, one of the two sterile access ports (medication ports) of the blood bags was entered with an injection needle, and six parts by weight of distilled, non-pyrogenic water were added for each one part by weight of cells present in the bag, for lysis of the cells. The weight of the cells can be determined conveniently by subtracting the standard dry weight of the blood bag from the actual weight of the specific bag and the cells contained therein.

The distilled water was agitated in the blood bag, and then allowed to stand for 24 hours at 4°C. Following this, the bag was centrifuged at a 1,000 Gravity force for seven minutes.

Following this, the liquid contents of each bag were passed through a 170 micron filter (a sterile Fenwal (Registered Trade Mark) in line filter set, available from the Fenwal Division of Baxter Laboratories, Inc., Morton Grove, Illinois), to separate them from the settled solids, and placed in a freezing environment until solidly frozen.

The frozen *Limulus* lysate solution was then carefully thawed, while assuring that the solution remained cold (i.e. below about 20°C.). After thawing, the *Limulus* lysate solution was prefiltered into a pooling bottle through an addition Fenwal 170 micron filter.

The filter residue remaining behind in the filter is unwanted material which has precipitated during the freezing step. The filtrate then is typically filtered once again through

another filter (a Millipore (Registered Trade Mark) type AP25 prefilter having a nominal pore size of 1.5 microns), followed by filtration with a Millipore membrane filter, stated to have an absolute pore size of 1.2 microns, and a nominal pore size of less than that. A nominal pore size is defined as that pore diameter which removes at least 98 per cent of all particles of the size stated.

Prior to use, all filters are rinsed with 1 liter of sterile, non-pyrogenic water. The last filtration steps proceed by pressurizing the lyzate solution upstream of the filter with approximately 2 lbs. of nitrogen gas pressure. Alternatively, vacuum in the collection vessel can be used to facilitate filtration.

After the last filtering step, the solution is subdivided into 2.0 ml. aliquots, which are placed in 6 ml. vials or test tubes, which have been thoroughly washed with sterile pyrogen-free water, and depyrogenated at 245°C for 4 hours. The test tubes are then conventionally sealed and shelf-frozen in a lyophilization machine (Virtis (Registered Trade Mark) Lyophilizer), and allowed to freeze-dry until a dry powder remains.

Reconstitution and Use of Limulus Lysate

A number of the test tubes prepared in the manner described above, were each reconstituted by the addition of 5 ml. of an equal volume mixture of 1 weight per cent magnesium chloride solution and 0.1 weight per cent sodium thioglycollate solution.

To calibrate the *Limulus* lysate, 0.1 ml. of non-pyrogenic water was placed into each of a series of empty test tubes, except the first tube. To the first tube was added 0.2 ml. of *E. coli* standard endotoxin solution (commercially available from Difco Laboratories, Detroit, Michigan). The concentration of the *E. coli* endotoxin used ranged from 100 nanograms of endotoxin per ml. Following this, 0.1 ml. of solution from the first tube was added to the second tube; and 0.1 ml. of solution from the second tube was added to the third tube; with this process being continued to form a series of successive test solutions each containing one-half of the concentration of the previous test solution, so that the twentieth and last test solution contains 0.00019 nanogram of endotoxin per ml. The sixth through the sixteenth numbers of this series were selected for use.

To several of the resulting tubes of solution there was added 0.1 ml. of the filtered lysate solution prepared above.

The series of tubes were then incubated at 37°C. for 60 minutes. Each tube was then inverted, and the presence or absence of an intact protein clot was noted.

Thereafter, all clots were broken up, and the tubes were centrifuged at 4,000 rpm. for 15 minutes. Following this, the supernatant liquid was removed from each tube, to remove dissolved protein, while any settled precipitate was allowed to remain in the tube.

To each tube was then added 0.2 ml. of 0.75 N sodium hydroxide solution to dissolve the protein precipitate.

Following this, the protein content of each vial was quantitatively analyzed in a manner similar to the procedure of Oliver H. Lowry, et al. as described in the article in *Journal of Biological Chemistry, Vol. 193, pages 265—275 (1951).*

The following reagents were used in the test described below:

Reagent A 20 grams/liter sodium carbonate in 0.1 N sodium hydroxide solution

Reagent B 5 grams/liter $CuSO_4 . 5H_2O$ in 10 grams/liter potassium tartrate solution

Reagent C alkaline copper solution made by mixing 49 ml. of Reagent A with 1 ml. of Reagent B. This material should be prepared fresh each day.

Reagent D diluted Folin reagent made by diluting of Folin-Ciocalteu phenol reagent (available from Fisher Scientific Co. or Harleco Company) diluted to 1 N with distilled water.

To each sample of protein solution in the vials prepared previously, one ml. of Reagent C was added. The mixture was thoroughly mixed and allowed to stand for about ten minutes at room temperature. Following this, 0.1 ml. of Reagent D was added rapidly and mixed within less than ten seconds, after which it was allowed to stand for at least thirty minutes at room temperature.

A Gilford 300 N spectrometer was set to measure absorbence on an optical density scale at a wavelength of 500 mμ. The spectrophotometer was equipped with a micro (0.5 ml.) flow-through cuvette to accommodate the small volume of the samples. The spectrophotometer setting may typically range between 450 and 1000 mμ but preferably is in the range of 500 and 750 mμ.

The spectrophotometer was adjusted to a zero absorbence reading with a blank standard comprising a solution of 0.75 N NaOH containing similar concentrations of solutions C and D. Under this technique, the concentration of lysate added is enough to give an absorbence reading in excess of 1 upon complete precipitation of the protein, and an absorbence reading of less than 0.2 in the essential absence of precipitation (and endotoxin).

Following this, the absorbence of samples previously prepared was measured in terms of optical density and recorded. The results are as indicated below:

TABLE I

Concentration of Endotoxin added to Lysate solution (nanograms per ml.)	Absorbence of precipitated protein	Calculated amount of precipitated protein (micrograms per 0.1 ml. Lysate) solution
3.12 (Solution No. 6)	1.031	425
1.56 (Solution No. 7)	1.110	475
0.78 (Solution No. 8)	1.386	(Absorbence is too high to accurately calculate protein).
0.39 (Solution No. 9)	0.941	370
0.195 (Solution No. 10)	0.913	350
0.097 (Solution No. 11)	0.958*	375
0.048 (Solution No. 12)	0.814	287
0.024 (Solution No. 13)	0.682	210
0.012 (Solution No. 14)	0.535	132
0.006 (Solution No. 15)	0.350	77.5
0.003 (Solution No. 16)	0.378	82.5
Control lysate solution with no added endotoxin	0.136	37.5

* This was the lowest concentration of endotoxin which was capable of forming a conventional protein clot positive reaction.

From the above Table 1 it can be seen that there is a sharp rise in the amount of protein precipitated in the range of solutions beginning with solution No. 14 and ending with solution No. 11. When one compares, in this series of solutions, the amount of protein precipitated by each member of the series with the next more dilute member of the series, there is noted a clear increase in the amount of protein precipitated. This is a clear indication that the increasing levels of endotoxin are causing increasing amounts of protein to precipitate, and constitutes typical behavior of purified *Limulus* protein in the presence of endotoxin.

Accordingly, it can be seen that the test of this Example is sensitive to the presence of endotoxin at concentration levels as low as 0.012 nanogram per ml. of test solution, since the protein precipitated in solution No. 14 is substantially greater in amount than the protein precipitated in solution No. 15. The endotoxin senisng reaction is confirmed by the further increase in precipitated protein by solutions 13, 12 and 11. One can also note that a suspected endotoxin reaction exists at concentrations as low as 0.003 nanogram per ml. (solution No. 16), since the precipitated protein level of even that extremely diluted sample is substantially above the control lysate solution having no added endotoxin.

Accordingly, when an unknown sample is tested under conditions identical to that described above, using an unused portion of the same lot of purified lysate solution, one can quantitatively analyze the concentration of pyrogen present, particularly endotoxin, by correlation with the absorbence reading for the unknown sample. For example, an unknown sample treated in accordance with the above Example which exhibits an absorbence reading of about 0.535 can be known to have an endotoxin concentration of approximately that of solution No. 14 in Table 1 above. Higher readings correspond to the higher endotoxin levels of the more concentrated solutions

as shown, although it will be noted that the readings appear to develop some quantitative error at the more concentrated levels (solution No. 11 and higher). However, these readings still retain rough accuracy, and also are usable for qualitative indications of the presence of endotoxin.

The above data is to be compared with the conventional clot formation technique of analysis, in which the presence of endotoxin is indicated by the formation of a clot in the reaction tube which does not break up when the test tube is carefully inverted by 180°. By this analysis technique, the most dilute solution in the above experiment which gave a positive endotoxin reaction was solution No. 11, having a concentration of 0.097 nanogram per ml. Accordingly, the above example provides an endotoxin determination which is eight times more sensitive than the conventional clot endotoxin determination.

Reference is made to co-pending application No. 16510/75 (Serial No. 1,459,733), which describes and claims:—

"A method of improving the endotoxin sensitivity of a solution of lysed *Limulus* blood cells, comprising freezing the solution, from which solid components such as cell wall fragments have been removed; thawing said solution; and thereafter filtering said solution through a filter having a nominal pore size (as hereinbefore defined) of no more than 2 microns, to remove components which are precipitated by said freezing step."

WHAT WE CLAIM IS:—

1. A method of analyzing a material having an unknown pyrogen content, comprising:—

A) Calibrating a purified solution of portions of *Limulus* lysate, which portions precipitate *Limulus* protein in the presence of a pyrogen, to determine the quantitative sensitivity of the solution to the presence of said pyrogen, said calibration comprising:—

(i) preparing a series of serially diluted dispersions of said pyrogen, wherein a plurality of these dispersions have concentrations of pyrogen of no more than 0.39 nanogram per ml.;

(ii) adding to said series of dispersions a given quantity of said purified *Limulus* lysate solution to form reaction mixtures; and

(iii) thereafter measuring, in a quantitative, photometric manner, the amount of *Limulus* protein precipitated, whereby an abrupt increase in the amount of precipitated protein in one member of said serially diluted series of dispersions when compared with the next more diluted member of said serially diluted series, indicates a positive pyrogen sensing reaction; and

B) Adding to said material said given quantity of a previously unused portion of said purified *Limulus* lysate solution and allowing the reaction mixture to stand under reaction conditions substantially equivalent to the reaction conditions under which purified *Limulus* lysate solution was added to each member of the serially diluted dispersions and thereafter measuring, in quantitative, photometric manner, the amount of protein precipitated in said material from said pyrogen precipitatable portions, whereby the precipitation of a concentration of protein which is at least equal to the concentration of protein precipitated in a said member of the serially diluted series of dispersions in which said abrupt increase in precipitated protein was noted constitutes an indication of a positive reaction to pyrogen in said material of unknown pyrogen content.

2. A method according to Claim 1, wherein said series of serially diluted dispersions of pyrogen include a plurality of dispersions having pyrogen concentrations of no more than 0.097 nanogram per ml.

3. A method according to Claim 1 or 2, in which said precipitated protein is measured by removing said precipitated protein from its supernatant solution from which it was precipitated, and thereafter quantitatively determining the amount of precipitated protein present by measuring the relative photo-absorbence, compared with a control protein, of a solution of said precipitated protein with Folin reagent at a wavelength between 450 and 1,000 millimicrons.

4. A method according to Claim 3, in which said wavelength is 500 to 750 millimicrons.

5. A method according to any preceding Claim, in which the amount of protein in each predetermined aliquot of purified solution which is added to each of said series of dispersions is sufficient to provide a photo-absorbence in the wavelength observed of less than 0.2 in the substantial absence of said pyrogen, and a photo-absorbence in the wavelength observed in excess of 1 in the presence of sufficient of said pyrogen to precipitate substantially all of said precipitatable protein.

6. A method according to any preceding Claim, in which the reaction mixture of each member of the serially diluted dispersions of pyrogen with said purified *Limulus* lysate solution is incubated.

7. A method according to any one of Claims 1 to 5, in which the reaction mixture of each member of the serially diluted dispersions of pyrogen with said purified *Limulus* lysate solution is incubated for 60 minutes at 37°C. before the measuring step is carried out on each member.

8. A method according to any preceding Claim, in which each member of the serially diluted dispersions of pyrogen has a pyrogen concentration one half of the concentration of the next more concentrated member in the series.

9. A method of analyzing a material having an unknown pyrogen content comprising cali-

brating a purified solution of portions of *Limulus* lysate, which portions precipitate *Limulus* protein in the presence of a pyrogen, to determine the quantitative sensitivity of the solution to the presence of said pyrogen and using the calibrated solution to test the material for pyrogen content, the method being carried out substantially as herein described.

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Printed for Her Majesty's Stationery Office by the Courier Press, Leamington Spa, 1978.
Published by the Patent Office, 25 Southampton Buildings, London, WC2A 1AY, from which copies may be obtained.